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Expression of TGF-β1 and matrix proteins is elevated in rats with chronic rejection

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Expression of TGF-β1 and matrix proteins is elevated in rats with chronic renal rejection. The pathogenesis of fibrosis in chronic renal allograft rejection remains unknown. Since TGF-β1 plays a key role in fibrogenesis, we studied a rat model of chronic allograft rejection that shows similarities to the structural lesion described in patients. We previously demonstrated an increased expression of TGF-β1 in human kidney biopsies with acute and chronic rejection. Recipients of renal allografts (F344-Lewis) and isografts (Lewis-Lewis) were sacrificed at 4, 8, 24 and 52 weeks. Characteristic histologic changes of chronic rejection developed in the allografts as early as four weeks and were accompanied by progressive albuminuria significant by eight weeks. Allografts showed a progressive increase in mRNA expression of TGF-β1 and matrix proteins during the 52 week course. Increased matrix deposition by immunofluorescence was mostly present in the interstitium and vessels early and in all kidney compartments later. The mRNA expression of plasminogen activator inhibitor, a protease inhibitor stimulated by TGF-β1, increased along with TGF-β1 and matrix proteins. These results suggest that the fibrosis of chronic renal allograft rejection is mediated, at least partly, by the dual action of TGF-β1 on matrix deposition and degradation.

Despite progressive improvements in the short-term outcome of transplantation, the annual rate of long-term graft loss has remained virtually unchanged [1, 2]. The most important cause of late graft attrition after the first year of transplantation is a poorly understood clinicopathological entity called "chronic rejection" and more correctly referred to as "chronic allograft nephropathy" since the exact nature of the process is unclear. It remains the single most important cause of graft loss after the first year [3–5]. Renal transplants with chronic rejection exhibit a gradual and progressive deterioration in function in association with proteinuria and hypertension [6]. The histology of chronic rejection in renal allografts is characterized by arterial intimal and medial fibrosis and arteriolar insudative lesions, glomerulosclerosis and interstitial fibrosis with tubular atrophy [7]. There is also a variable amount of tissue infiltration with T cells and macrophages [8, 9].

The pathogenesis of chronic rejection remains unknown [3–5]. Recent studies have shown that acute rejection is an important risk factor for chronic rejection [10, 11]. The role of HLA matching was also advanced as supporting an immunologic basis since the incidence of chronic rejection is lower in grafts from living related donors [12]. However, those grafts usually have a short ischemia time and immediate graft function which correlate with a better outcome. Although intense immunosuppression is useful in reversing acute rejection, it is ineffective in chronic rejection [13]. As a result, other non-immunological factors are thought to operate in the progressive deterioration of the allograft [14–16]. Independent of initiating events, the cause of tissue destruction in chronic rejection is fibrogenesis, or accumulation of extracellular matrix (ECM), which also occurs in chronic diseases of the kidneys and other organs [17–19]. Excess ECM accumulation ultimately leads to loss of allograft renal function.

The lack of a reproducible animal model of chronic rejection has hampered the study of the mechanisms involved in this disease. Recently, a previously described model of chronic renal rejection was reproduced and adapted [20]. Striking similarities between this model and the changes observed in the grafts of many patients with chronic rejection were reported [21]. A mononuclear cellular infiltrate was also shown to be present. A number of cytokines, presumably derived from this cellular infiltrate, were shown to be expressed suggesting an immunologic role for the development of chronic rejection in this model [22, 23]. However, nephron supply also seems to play a role in long term outcome in this model [24]. In addition, if allografts are retransplanted into the donor within 12 weeks, the histologic changes are reversed indicating that, while immunologic factors are operating early on, they do not play a role in the later stages of the disease [25].

To date, no study has looked at the components of the fibrotic lesion of chronic rejection in this model and none has attempted to correlate the renal fibrosis or the expression of ECM components with any growth factor. Since transforming growth factor-β (TGF-β) is a key fibrogenic cytokine, we hypothesized that TGF-β overexpression might be responsible for the fibrosis of chronic rejection in this model. TGF-β has been implicated in the fibrosis of a number of chronic diseases of the kidney and other organs [26, 27]. TGF-β directly stimulates the synthesis of individual ECM components [28–31]. It also blocks ECM degradation by decreasing the synthesis of proteases and stimulating protease inhibitors like plasminogen activator inhibitor-1 (PAI-1) [32–34]. In addition to its effect on matrix accumulation, TGF-β also promotes immune suppression [26, 35, 36].

Our findings in this paper indicate that all representative components of the ECM are elevated in this model and that their expression correlates with an increased expression of TGF-β1 and

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PAI-1. These results suggest that the ECM accumulation observed in this model of chronic renal allograft rejection likely involves the dual action of TGF-β on increasing ECM deposition and decreasing ECM degradation.

Methods

Experimental design

Inbred adult male rats, weighing 250 to 300 g were used in all experiments (Charles River, Wilmington, MA, USA). Lewis rats (LEW $RT1^1$) acted as recipients for the allografts and as donors and recipients for the isografts. Fisher rats (F344, $RT1^{1U1}$) acted as donors for the allografts. These rats differ partially at MHC classes I and II, and various non-MHC genes. Donor rats were anesthetized with an i.p. injection of chloral hydrate (300 mg/kg). The left kidney, left ureter and dome of the bladder were removed, then flushed and cooled in cold Collins' solution. The kidney was then positioned orthotopically in anesthetized, weight-matched Lewis recipients after the recipient left kidney vessels were mobilized, clamped and the native kidney excised. The donor and recipient renal artery, vein and ureter were each then anastomosed end-to-end with 10-0 prolene sutures without ureteral stenting [37]. The contralateral right native kidney was removed at day 10. All animals (allografts and isografts) received a short course of cyclosporine A 5 mg/kg/day (Sandimmune®; Sandoz Research Institute, East Hanover, NJ, USA) i.m. for 10 days after transplant to prevent early rejection which would otherwise prove fatal. All recipients received a single i.m. injection of 100,000 units of penicillin G (Pfizer, New York, NY, USA) at the time of grafting as prophylaxis for wound infection. Functional studies were done at 2, 4, 8, 12, 16, 24 and 52 weeks (see below) and animals were sacrificed at 4, 8, 24 and 52 weeks ($N = 5$ in each group). Animals were anesthetized with ketamine and the abdomen was opened through a midline incision. With the aorta occluded by ligation above the renal artery and the renal vein opened by a small incision for outflow, the transplanted kidney was perfused with 20 ml of cold heparinized saline. The kidney was removed, the cortex was carefully dissected from the medulla, and tissues were processed for evaluation by light microscopy, RNA analysis and immunohistochemistry.

Functional studies

Blood was sampled from the rat tail vein and 24-hour quantitative urine samples were collected in metabolic cages (Nalge Co., Rochester, NY, USA). Serum and urine creatinine were determined colorimetrically by using creatinine reagents (Sigma Chemical Co., St. Louis, MO, USA) after appropriate dilutions were done. To determine urine albumin, an inhibitory ELISA technique modified from the method of Rennard et al was used [38]. Wells in one microtiter plate were coated with rat serum albumin (Sigma Co., St. Louis, MO, USA). To another plate, known serial dilutions of serum albumin or unknown samples and rabbit IgG anti-rat albumin (Cappel, Durham, NC, USA) were added. After washing the coated plate, aliquots of the second plate were added to the coated plate and incubated. After washing, horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL, USA) was added and developed using standard methods. The absorption of the colored reaction product was then measured in a microtiter plate reader (Molecular Devices, Menlo Park, CA, USA) at 450 nm. The creatinine clearance was calculated using a standard formula.

Morphology

Renal tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Two to four micron thick sections were stained with periodic acid-Schiff's reagent (PAS) and Trichrome. The histologic findings were subdivided into three categories: glomerulosclerosis, tubulointerstitial fibrosis and arteriolopathy. Glomerulosclerosis was defined by increased mesangial matrix without increased cellularity and also loss of capillary patency. Features of interstitial inflammation were mononuclear and lymphocytic infiltrates and edema. Findings of scarring were matrix-rich expansion of the interstitium with distortion of the tubules and thickening of the tubular basement membranes. Renal arterial and arteriolar lesions were characterized by hyalin deposition within the tunica media of arterioles, hypertrophy of the vessel wall, and perivascular cellular infiltrates and fibrosis. A minimum of 20 fields at 100× and 250× magnifications were assessed and graded with a minimum of two sections in each biopsy by an observer masked to treatment groups using a color image analyzer (Carl Zeiss, Inc., Oberkochen, Germany).

The following semiquantitative score was used to assess the extent of changes in each category. For glomerulosclerosis, the percentage of each glomerulus occupied by mesangial matrix and the number of obliterated capillary lumina was estimated and assigned a semi-quantitative score using the following: 0 = < 25% glomerulosclerosis, 1 = 25 to 50%, 2 = 50 to 75% and 3 = 75 to 100%. Tubulointerstitial fibrosis was estimated by assigning the following scores: 0 = normal interstitium and tubules, 1 = mild to moderate fibrosis with mild interstitial thickening between tubules, 2 = moderate fibrosis with interstitial thickening between tubules and minimal tubular dilation, 3 = severe fibrosis with tubular dilation, significant interstitial thickening between tubules and tubular basement membrane thickening. Arteriolopathy was semiquantitatively determined by the following: 0 = < 15% arterioles injured, 1 = 15 to 30% arterioles injured, 2 = 31 to 75% and 3 = > 75% injured.

RNA analysis

After separating cortex from medulla, the tissue was finely minced with a razor blade on ice. Total RNA was prepared by lysis in 4 M guanidine isothiocyanate containing 1% 2-mercaptoethanol and 0.5% lauryl sarcosyl and ultracentrifugation of the lysate on a cesium chloride cushion. After resuspending in Tris-EDTA buffer, RNA concentrations were determined using spectrophotometric readings at $Absorbance_{260}$. Thirty micrograms of RNA were electrophoresed in each lane of 0.9% agarose gels containing 2.2 M formaldehyde and 0.2 M Mops (pH 7.0) and transferred to a nylon membrane (ICN Biochemical, Inc., Costa Mesa, CA, USA) overnight by capillary blotting. Nucleic acids were crosslinked by ultraviolet irradiation (Stratagene, La Jolla, CA, USA). The membranes were prehybridized for two hours at 42°C with 50% formamide, 10% Denhardt's solution, 0.1% SDS, 5× standard saline citrate (SSC), and 200 μg/ml denatured salmon sperm DNA. They were then hybridized at 42°C for 18 hours with cDNA probes labeled with ^{32}P-dCTP by random oligonucleotide priming (Boehringer Mannheim Corp., Indianapolis, IN, USA). The blots were washed in 2 × SSC, 0.1% SDS at room temperature for 15 minutes and in 0.1 × SSC, 0.1% SDS at 50°C for 15

Table 1. Serial serum creatinine values during the 52 week model

Weeks after transplantation	Allografts	Isografts	*P* value
2	0.6 ± 0.1	0.4 ± 0.1	NS
4	0.8 ± 0.2	0.4 ± 0.1	< 0.05
8	0.8 ± 0.2	0.4 ± 0.1	NS
12	0.6 ± 0.2	0.4 ± 0.1	< 0.05
16	0.6 ± 0.2	0.4 ± 0.1	NS
24	0.6 ± 0.1	0.5 ± 0.1	NS
52	2.1 ± 0.1	0.6 ± 0.0	< 0.001

Values are mean ± SEM of 5 rats.

minutes. Films were exposed at −70°C for different time periods to ensure linearity of densitometric values and exposure time. Autoradiographs were scanned on a laser densitometer (Ultrascan XL; Pharmacia LKB Biotechnology, Inc., Bromma, Sweden). The density of bands for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to control for differences in the total amount of RNA loaded onto each gel line. For quantitative purposes, the values were divided by the density of bands for GAPDH in the same lane. The following probes were used for Northern blot analysis.

A mouse TGF-β1 cDNA probe (plasmid MUI5) was kindly provided by R. Derynck [39]. A rat PAI-1 cDNA probe [plasmid pBluescript SK(-)] was obtained from T.D. Gelehrter [40]. Plasmid P16, which contains a human biglycan insert, was a gift of L.W. Fisher [41]. A rat decorin cDNA probe (plasmid pGEM4) was provided by K.L. Dreher [42]. A rat procollagen α1 cDNA (plasmid pa1R1) was obtained from D. Rowe [43]. A rat GAPDH cDNA probe (plasmid pBluescript KS II) was a generous gift from J.M. Blanchard [44].

Immunohistochemistry

Immunofluorescence microscopy was performed on tissues snap frozen in cold isopentane with a cryostat microtome (Miles Scientific, Naperville, IL, USA) as previously described [45]. Four micrometer cryostat sections were fixed in acetone and washed in PBS, pH 7.4. The deposition of the following ECM components, decorin, tenascin and fibronectin-cellular containing extra domain A (fibronectin EDA+) was determined. The primary antibody for decorin was a rabbit anti-human decorin antibody and was kindly provided by E. Ruoslahti (La Jolla, CA, USA) [46]. The other primary antibodies used were rabbit anti-human tenascin (Life Technologies, Gaithersburg, MD, USA) and mouse anti-human fibronectin EDA+ (Sera-lab, Crowley Down, UK). The secondary antibodies used were fluorescein isothiocyanate (FITC)-conjugated F(ab')2 donkey anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA) and FITC-conjugated F(ab')2 rat anti-mouse IgG (Jackson Immunoresearch). A minimum of 20 randomly selected areas per sample were observed at ×250 magnification. The severity of staining for the glomerular, tubulointerstitial and vascular compartments was evaluated by an observer blinded to the treatment groups using the following semiquantitative scale: 0 = diffuse, very weak or absent staining, 1 = staining involving less than 25%, 2 = staining involving 25% to 50%, 3 = staining involving 50% to 75% and 4 = staining involving 75% to 100%. Photographs were obtained at identical exposure and development time intervals.

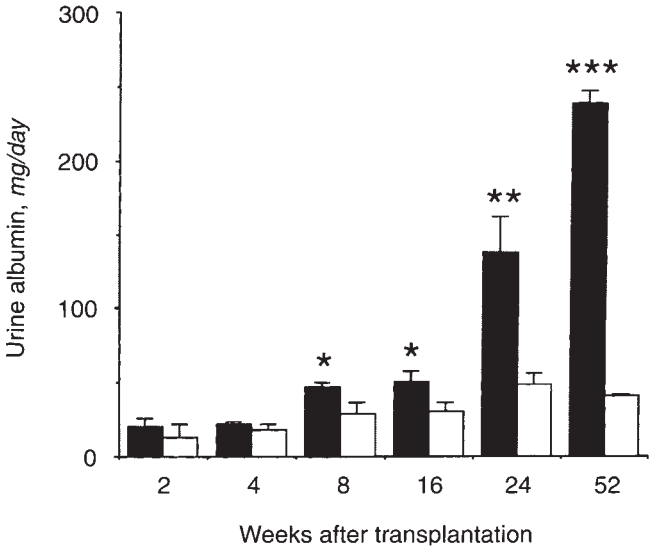

Fig. 1. *Urine albumin excretion in both allografts (■) and isografts (□) during the course of this 52-week model.* Significant differences in albuminuria between isografts and allografts were evident as early as eight weeks (29.0 vs. 46.8 mg/day, respectively). By 24 weeks, there was nearly a threefold increase in albuminuria in allografts (138.2 vs. 48.4 mg/day in isografts) and by 52 weeks, albuminuria had risen to 238.7 mg/day in allografts versus 41.0 mg/day in isografts, which represents a sixfold increase over controls. $N = 3$ to 5 for each group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to isografts.

Statistical analysis

Results are presented as mean ± SEM. Multiple comparisons were done by analysis of variance. Comparisons between allografts and isografts were done by two-tailed unpaired Student's *t*-test or Mann-Whitney test, as appropriate. The level of statistical significance was chosen as $P < 0.05$.

Results

Physiologic studies

Body weight gain was progressive and comparable in the allografts and isografts throughout the study. Changes in body wt between the start of the experiment and sacrifice remained similar in control and experimental groups and did not achieve statistical significance. Serum creatinine level was significantly elevated at 4 and 12 weeks in the allografted rats (0.8 ± 0.2 vs. 0.3 ± 0.1 mg/dl, $P < 0.05$, 4 weeks; 0.6 ± 0.2 vs. 0.3 ± 0.1, $P < 0.05$, 12 weeks). However, it remained equivalent between the two groups for the duration of the study at weeks 16, 20 and 24 and did not become again statistically significant ($P < 0.001$) until 52 weeks when it rose to 2.1 ± 0.1 mg/dl in the allografts versus 0.5 ± 0.0 mg in the isografts (Table 1). Creatinine clearance was calculated at 2, 4, 8, 24 and 52 weeks and was consistently worse in the allografts without reaching, however, statistical significance until 52 weeks ($P < 0.001$) (data not shown). Significant differences in albumin excretion between isograft and allograft recipients were evident as early as eight weeks after transplant (29.0 vs. 46.8 mg/day, respectively, $P < 0.05$; Fig. 1). By 24 weeks, there was nearly a threefold increase in urine albumin excretion in the allografts (138.2 vs. 48.4 mg/day, $P < 0.05$) and by 52 weeks, urine albumin excretion had risen to 239 mg/day in the allografts versus 41 mg/day in the isografts ($P < 0.001$), which represents a sixfold

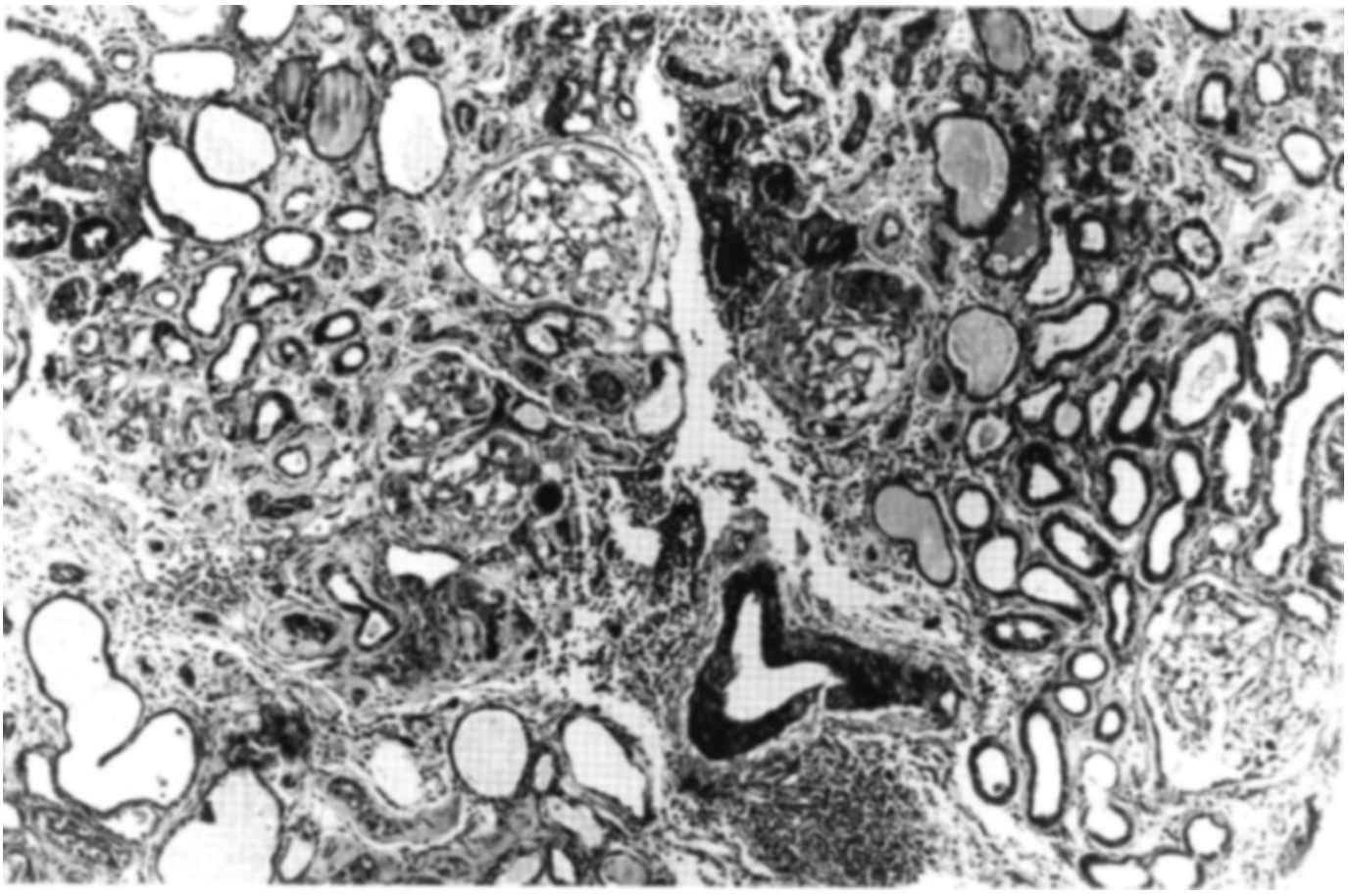

Fig. 2. *Histologic changes in experimental chronic renal allograft rejection.* Micrograph showing the renal cortex of an allograft sacrificed at 52 weeks after transplantation. There is evidence of segmental glomerulosclerosis and interstitial fibrosis with tubular atrophy. A mononuclear cellular infiltrate is also observed. The vessel shown is mildly thickened with perivascular fibrosis (periodic acid-Schiff, magnification ×10).

increase over control. These values are consistently higher than values observed in age-matched normal Lewis rats whose urine albumin excretion can vary between 0.5 and 1.5 mg/day.

Histologic changes

The allografts had characteristic morphologic findings similar to the human chronic rejection lesion. These changes were mostly present by 24 weeks (Fig. 2) but were evident as early as four weeks after engraftment. Allografts exhibited a gradual and significant increase in segmental and global glomerulosclerosis. There was progressive tubulointerstitial fibrosis with tubular atrophy associated with thickening of the tubular basement membrane and Bowman's capsule. In addition, a variable degree of interstitial mononuclear cell infiltrate was observed. The vascular lesion was characterized by hyalin deposition within arterioles, hypertrophy of vessel walls, and perivascular cellular infiltrates and fibrosis. The extent of changes was graded using a 0 to 3+ semiquantitative scale with 3+ indicating extensive changes. Table 2 summarizes the histological scoring observed in this model. Histologic changes of chronic rejection in the allografts were evident as early as four weeks after transplantation and remained significant when compared to the isografts throughout the study period. Between weeks 16 and 52, a relatively high mortality rate in the allograft rats was observed in our study probably because of worsening histology. As a result, only the less diseased allograft rats were studied. We observed a progressive glomerulosclerosis in the isografts in a manner similar to what has been described in aging and uninephrectomized rats. This progressive glomerulosclerosis in the isografts, coupled with the high mortality rate observed in the allografts, may explain the lack of significant glomerular changes at 52 weeks when comparing the allografts to the isografts. However, significant glomerulosclerosis was observed in the allografts versus the isografts as early as four

Table 2. Semiquantitative scoring by light microscopy of the extent of changes observed in the chronic allograft and isograft kidneys for glomerulosclerosis, tubulointerstitial fibrosis and arteriolopathy

	Glomerulosclerosis		Tubulointerstitial fibrosis		Arteriolopathy	
Week	A	I	A	I	A	I
4	0.6 ± 0.0[c]	0.0 ± 0.0	2.8 ± 0.5[a]	0.4 ± 0.1	1.3 ± 0.3[b]	0.4 ± 0.2
8	0.6 ± 0.0[a]	0.1 ± 0.1	2.9 ± 0.1[b]	0.5 ± 0.4	1.2 ± 0.3[b]	0.3 ± 0.0
24	1.1 ± 0.2[b]	0.3 ± 0.2	2.6 ± 0.2[b]	0.2 ± 0.2	1.2 ± 0.1[c]	0.1 ± 0.1
52	1.1 ± 0.3	1.0 ± 0.1	3.7 ± 0.1[c]	0.4 ± 0.1	1.5 ± 0.1[b]	0.4 ± 0.1

Values are mean ± SEM of 5 rats. Abbreviations are: A, allografts; I, isografts.

[a] $P < 0.05$, [b] $P < 0.01$ and [c] $P < 0.001$ vs. isografts

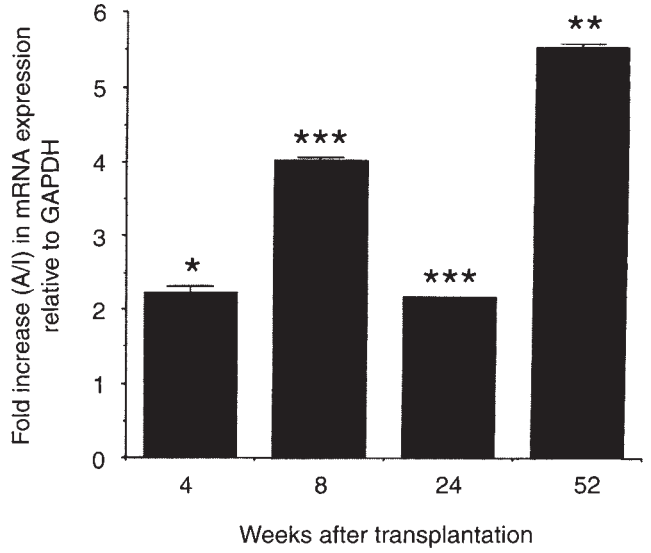

Fig. 3. *Quantitation of mRNA expression of TGF-β1 in allografts relative to isografts.* Total RNA was isolated from whole cortex at weeks 4, 8, 24 and 52 and was hybridized with cDNA probes to TGF-β1. Band densities for GAPDH mRNA were used to control for differences in total amount of RNA loaded onto each gel line. Values obtained for allografts were then divided by values obtained for isografts and expressed as fold-increase in mRNA. $N = 5$ for each group, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to isografts. Abbreviations are: A, allografts; I, isografts.

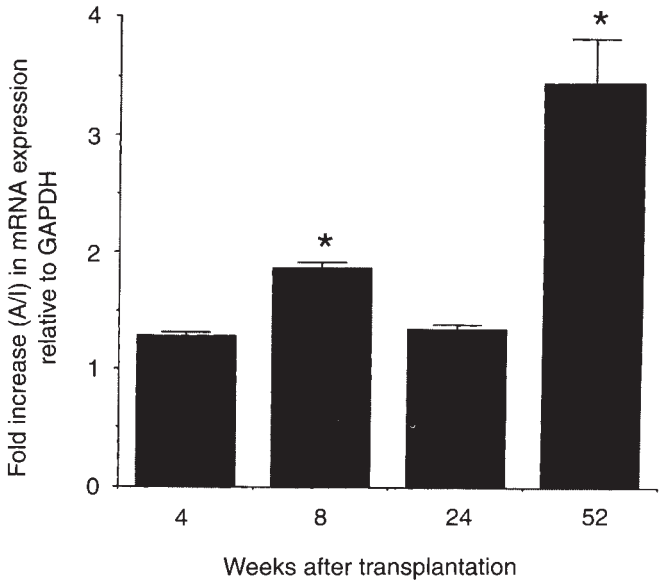

Fig. 4. *Quantitation of mRNA expression of PAI-1 in allografts relative to isografts.* Total RNA was isolated from whole cortex at weeks 4, 8, 24 and 52 and was hybridized with cDNA probes to PAI-1. Band densities for GAPDH mRNA were used to control for differences in total amount of RNA loaded onto each gel line. Values obtained for allografts were then divided by values obtained for isografts and expressed as fold-increase in mRNA. $N = 5$ for each group, $^{***}P < 0.001$ compared to isografts. Abbreviations are: A, allografts; I, isografts.

weeks ($P < 0.001$) and up to 24 weeks ($P < 0.001$). On the other hand, the degree of tubulointerstitial fibrosis and arteriolopathy remained significant throughout the study period ($P < 0.05$ at weeks 4 and 8, < 0.001 at week 24 and < 0.01 at week 52) when compared to isografts.

Expression of TGF-β1 and PAI-1 mRNA

The expression of TGF-β1 mRNA in the cortex of the allografts was elevated by week 4 ($P < 0.05$) when compared to isografts and more so by weeks 8 and 24 ($P < 0.001$) and week 52 ($P < 0.01$; Fig. 3). The mRNA expression of PAI-1, a protease inhibitor that blocks ECM degradation by the plasmin protease system and that is directly stimulated by TGF-β, is shown in Figure 4. PAI-1 mRNA, in parallel to TGF-β1 mRNA expression, was consistently up-regulated in allografts as compared to isografts but reached statistical significance only at 8 ($P < 0.001$) and 52 weeks ($P < 0.05$).

Extracellular matrix deposition in the kidney

Northern blot analysis. The proteoglycans biglycan and decorin are components of the ECM directly regulated by TGF-β. Their mRNA expression was elevated suggesting active matrix synthesis (Fig. 5). Biglycan mRNA followed closely TGF-β1 mRNA expression and was significantly elevated both in the early follow-up period at weeks 4 ($P < 0.05$) and 8 ($P < 0.001$), and later at weeks 24 ($P < 0.01$) and 52 ($P < 0.05$). The mRNA expression of decorin (Fig. 5) was also statistically elevated in the allografts in a manner similar to that of biglycan (week 4, $P < 0.05$; weeks 8, 24 and 52, $P < 0.01$). Type I collagen is normally present in the interstitium of the kidney and is directly increased by TGF-β. In this model, the mRNA expression of type I collagen (Fig. 6) was similar to TGF-β1 and PAI-1 mRNA expression and was increased at all time periods, with its expression reaching statistical significance at weeks 4 ($P < 0.01$), 8 ($P < 0.001$) and 52 ($P < 0.01$) in the allografts versus isografts.

Immunohistochemistry. To assess whether the increased mRNA expression was translated into increased synthesis and deposition of matrix proteins, certain components of the ECM were identified by immunofluorescence. This also allowed us to determine which kidney compartments (glomeruli, vessels or tubulointerstitium) were most extensively involved in matrix expansion. We examined the expression of tenascin, fibronectin EDA+, and decorin. The amount of tenascin and fibronectin EDA+ was elevated in the allografts as compared to isografts in all kidney compartments and at all time intervals (Figs. 7 and 8). The changes were most significant in the tubulointerstitium for both tenascin ($P < 0.01$ at weeks 4 and 24 and < 0.001 at weeks 8 and 52) and fibronectin EDA+ ($P < 0.01$ at weeks 4, 8 and 24 and <0.05 at week 52; Fig. 8). The observed arteriolar hyalinosis correlated with a progressive and significant increase in the expression of tenascin and fibronectin EDA+ in the vessels of the allografts. While it was not significant at four weeks, it reached statistical significance at weeks 8 ($P < 0.001$ for tenascin and < 0.05 for fibronectin EDA+), 24 ($P < 0.01$) and 52 ($P < 0.01$) when compared to isografts (Fig. 8). Most of the changes reached statistical significance in the tubulointerstitial and vascular compartments of the kidney in accordance with the characteristic histology of chronic allograft rejection. In the glomeruli, none of the changes were statistically significant except early at 4 weeks ($P < 0.01$) and later at 52 weeks ($P < 0.001$; Fig. 8).

The deposition of decorin followed its mRNA expression and that of tenascin and fibronectin EDA+ (Figs. 7 and 8). Most of

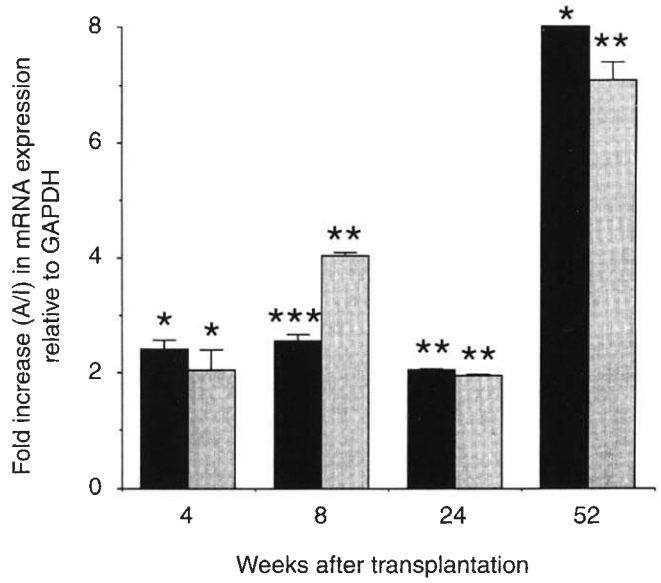

Fig. 5. *Quantitation of mRNA expression of the proteoglycans, biglycan (■) and decorin (▨) in allografts relative to isografts.* Total RNA was isolated from whole cortex at weeks 4, 8, 24 and 52 and was hybridized with cDNA probes to biglycan and decorin. Band densities for GAPDH mRNA were used to control for differences in total amount of RNA loaded onto each gel line. Values obtained for allografts were then divided by values obtained for isografts and expressed as fold-increase in mRNA. $N = 5$ for each group, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to isografts. Abbreviations are: A, allografts; I, isografts.

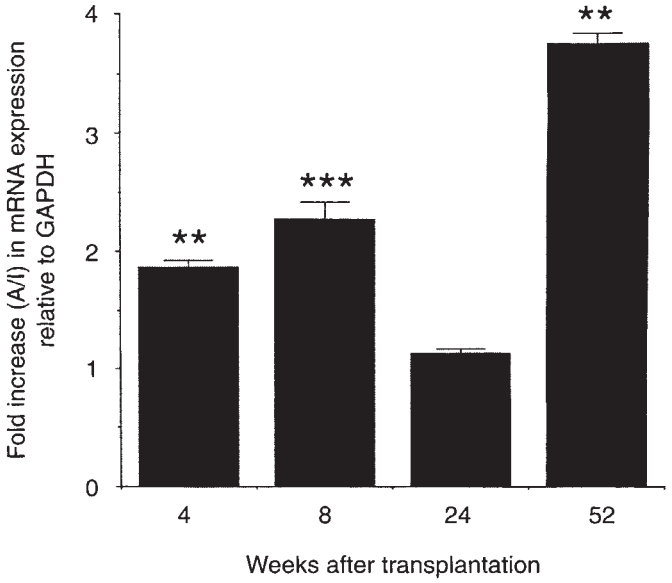

Fig. 6. *Quantitation of mRNA expression of type I collagen in allografts relative to isografts.* Total RNA was isolated from whole cortex at weeks 4, 8, 24 and 52 and was hybridized with cDNA probes to type I collagen. Band densities for GAPDH mRNA were used to control for differences in total amount of RNA loaded onto each gel line. Values obtained for allografts were then divided by values obtained for isografts and expressed as fold-increase in mRNA. $N = 5$ for each group, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to isografts. Abbreviations are: A, allografts; I, isografts.

the changes in the allografts were observed at 24 and 52 weeks ($P < 0.01$) but were statistically significant also at 4 and 8 weeks ($P < 0.05$) when compared to the isografts.

Discussion

We have previously shown an elevated expression of TGF-β isoforms and fibronectin EDA+ in human renal allograft biopsies with both acute and chronic rejection, and suggested that TGF-β plays a role in the fibrosis of human chronic rejection [45]. In this animal model of experimental chronic renal allograft rejection, our findings demonstrate that the expression of TGF-β1 and different matrix components is also increased. Specifically, we detect an elevated mRNA expression of the proteoglycans biglycan and decorin and of type I collagen, and an increased deposition, by immunofluorescence, of the glycoproteins tenascin and fibronectin EDA+, and the proteoglycan decorin. TGF-β1 has been shown to directly induce the production of all these matrix components [27–31]. Our results therefore suggest that the increased TGF-β1 expression leads to matrix accumulation. In addition, we demonstrated an elevated mRNA expression of the protease inhibitor PAI-1. The plasmin system is involved in matrix degradation and is inhibited by PAI-1. TGF-β1 has been shown to block matrix degradation by inducing PAI-1 production [32, 33]. As a result, inhibition of matrix degradation represents an additional mechanism through which TGF-β leads to matrix accumulation in this model. We have previously shown that a similar mechanism of increased matrix production and decreased degradation under the influence of TGF-β1 leads to matrix accumulation in experimental chronic cyclosporine nephrotoxicity [47].

The observed fibrosis in this model was associated with an increased deposition of the three major components of the ECM: proteoglycans, glycoproteins, and collagens. Matrix deposition was initially most marked in the interstitium and vessels. However, by 24 weeks, the changes were equally severe in all compartments of the kidney, including a progressive increase in the glomerular lesion and increased albuminuria. Significant albuminuria developed by eight weeks in the allografts and progressed during the 52 weeks of observation. The isografts developed only a mild and progressive degree of albuminuria and segmental glomerulosclerosis. This is similar to renal ablation models in rats where a simple reduction in renal mass has been shown to lead to progressive albuminuria and glomerulosclerosis [48]. The model in this study was recently successfully reproduced and characterized by a number of investigators [21–25]. While Diamond et al [21] showed a predominant glomerular lesion, we found not only glomerular, but also tubulointerstitial and vascular changes, similar to the human lesion of chronic rejection. The difference may be related to variations in histocompatibility between breeding stocks among different commercial suppliers.

Although not conclusive, the findings presented here, along with our previous results, suggest that TGF-β plays a causative role in the matrix expansion that occurs in both human and experimental chronic rejection. Matrix components induced by TGF-β, such as fibronectin EDA+ and tenascin, are preferentially expressed in healing wounds and are up-regulated in this model [49, 50]. Although TGF-β1 was previously shown to be expressed in chronic rejection [22, 51], this is the first report to demonstrate direct involvement of a growth factor in the deposition of ECM in experimental chronic rejection. A key feature of wound repair is the necessity to terminate the process to avoid

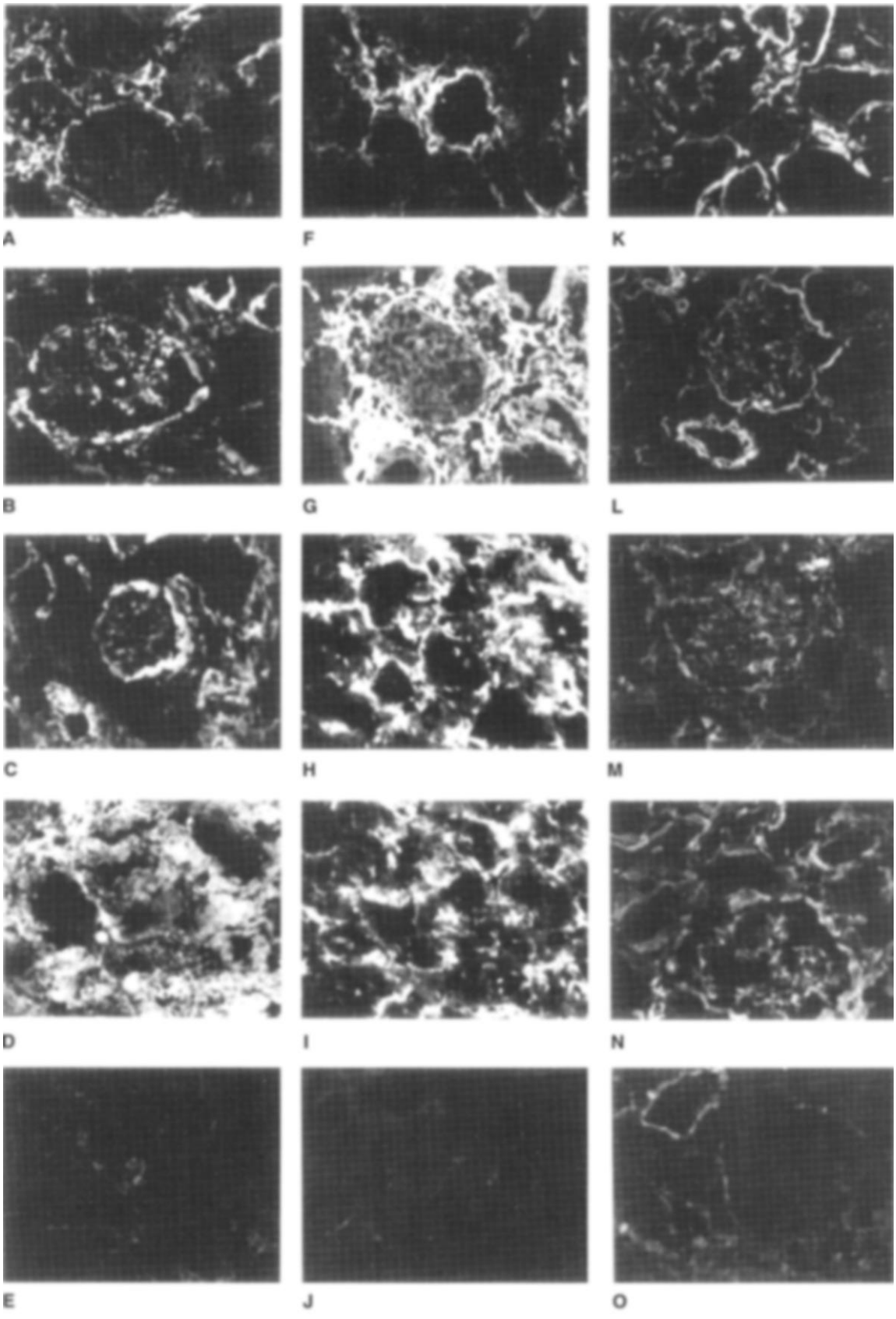

Fig. 7. *Immunofluorescence micrographs of the expression of the glycoproteins tenascin and fibronectin and the proteoglycan decorin in allografts and isografts.* Kidney sections from allografts (**A** to **D**, **F** to **I**, and **K** to **N**) and isografts (**E**, **J**, **O**) obtained at weeks 4 (A, F, K), 8 (B, G, L), 24 (C, E, H, J, M, O) and 52 (D, I, N) were stained with an antibody to tenascin (A to E), fibronectin EDA+ (F to J), and decorin (K to O). Photographs were taken under identical conditions with equal exposures of 60 seconds. Magnification ×250.

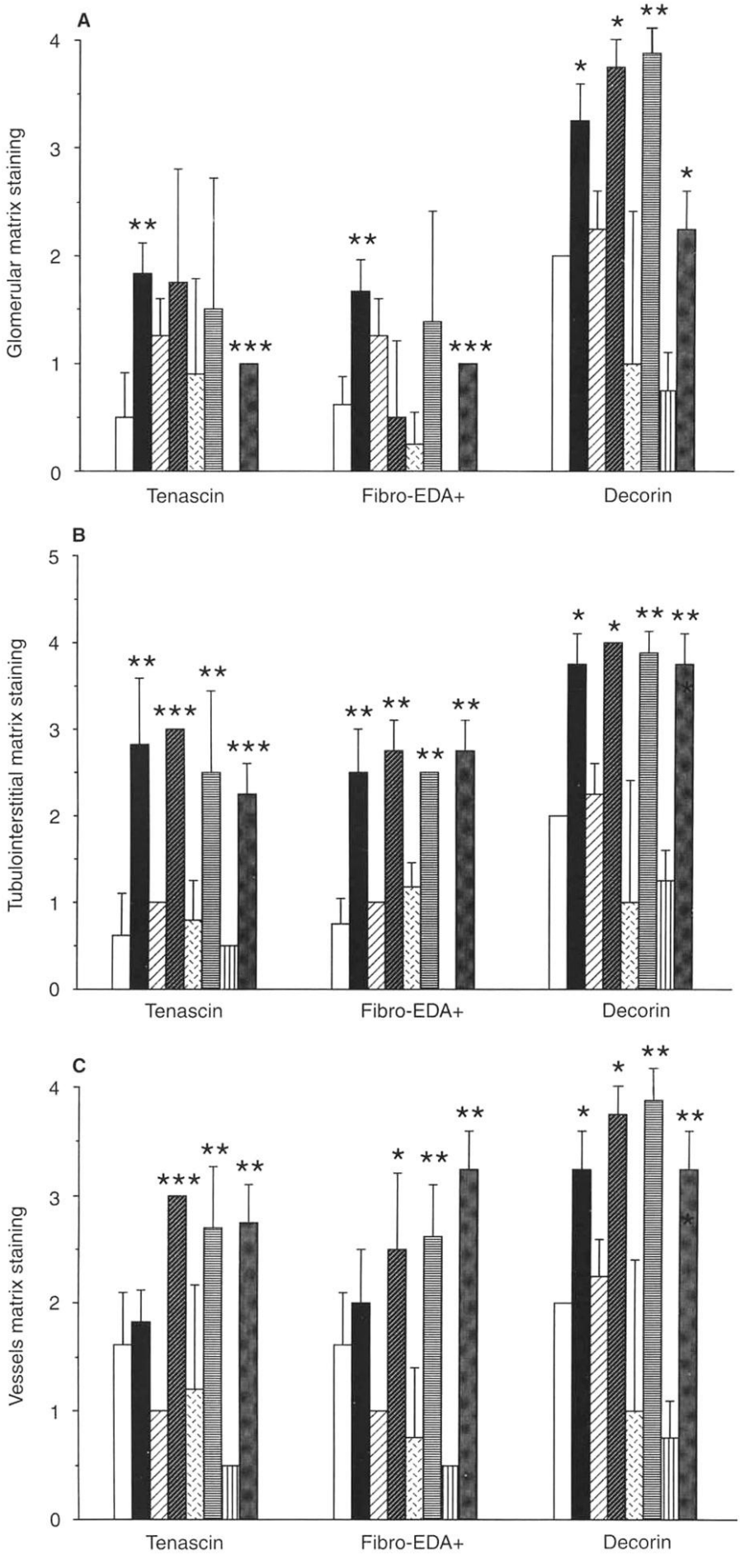

Fig. 8. *Quantitation of deposition of extracellular matrix in allografts and isografts by immunofluorescence.* (**A**) Glomerular staining, (**B**) tubulointerstitial staining, and (**C**) vessels staining score at weeks 4, 8, 24 and 52. Abbreviation is fibronectin EDA+, Fibro-EDA+. Symbols are: (□) isografts 4 weeks; (■) allografts 4 weeks; (▨) isografts 8 weeks; (▨) allografts 8 weeks; (▩) isografts 24 weeks; (▤) allografts 24 weeks; (▥) isografts 52 weeks; (▦) allografts 52 weeks; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to isografts.

excessive scarring [52]. The development of fibrosis in chronic renal allograft rejection suggests a failure to regulate or terminate the process of tissue repair, resulting in continued activity of TGF-β. TGF-β is known to induce its own production [53], and its persistent expression following repeated injuries leads, via positive feedback, to a cycle of continued TGF-β production [27]. Since TGF-β was elevated in both acute and chronic rejection, we previously suggested that persistent up-regulation of TGF-β, a result of acute rejection episodes, predisposes the graft to a cycle of sustained TGF-β expression and mediates the fibrosis of chronic rejection [47]. It is likely that a similar mechanism operates in this model in which an unabated TGF-β1 overexpression may be responsible for excess matrix deposition and fibrosis.

Because the etiology of chronic rejection is likely multifactorial, our study focused on the factors responsible for the progression of fibrosis. There are data to suggest that both immunological and non-immunological processes contribute to the etiology of chronic rejection [21–25]. What causes the elevation of TGF-β in this model remains unclear. When considering TGF-β in relation to allograft pathology, two aspects of its biological properties are particularly relevant: first, the ability of TGF-β to regulate ECM production [28–34], and second, the immunomodulatory functions of TGF-β. TGF-β is thought to be involved in the inflammatory response from onset to resolution and has important immunosuppressive actions [26, 35, 36]. These immunosuppressive activities are likely to explain the excessive inflammation that results in the absence of the TGF-β1 gene [36]. We can speculate that TGF-β expression may be increased in this model, not only as a result of injury, but also as a reflection of the donor or graft cells to attempt to induce graft tolerance. However, a number of factors may also influence the development of chronic rejection in this model including ischemia time, decreased nephron mass, disorders of lipoprotein metabolism, and the nephrotoxic effects of cyclosporine [14, 25, 47, 54]. In this 52 week model, cyclosporine was administered for only 10 days, which is a relatively short period of time for chronic cyclosporine nephrotoxicity to develop. As a result, its contribution to fibrosis seems unlikely, although it cannot be definitely ruled out.

In summary, our data suggest that the observed ECM accumulation in this model of chronic rejection likely involves the dual action of TGF-β1, not only on ECM deposition, but also on ECM degradation. It is unknown whether blocking TGF-β overexpression with specific antagonists can ameliorate the fibrotic lesion of chronic rejection and improve renal allograft function. Future studies using TGF-β antagonists will help elucidate this important question.

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